DIFFERENTIATION OF HEMOLYTIC STREPTOCOCCI FROM HUMAN AND BOVINE SOURCES BY THE HYDROLYSIS OF SODIUM HIPPURATE

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The close resemblance between hemolytic streptococci of human and bovine origin has led to extensive studies of these organisms. Hemolytic streptococci are commonly found in the udders of cows and are therefore present in the milk from such animals. Since these streptococci are so similar to the human hemolytic types in cultural characteristics, varying practically only in the vigor of their growth, milk containing them has been looked on as a potential source of infection for human beings.

The appearance of hemolytic colonies gives occasion for alarm to those familiar with the use of blood plates in connection with the examination of material from pathologic sources but unaccustomed to the plating of fresh milk on blood agar. However, those who have observed large numbers of samples of fresh milk plated on blood agar have been impressed with the fact that the hemolytic streptococci commonly found must be different from the human types. If such were not the case streptococcal infection from milk might be very widespread.

Hemolytic streptococci from the udder of the cow are mentioned specially because there are types found in pasteurized milk which are somewhat hemolytic, but which because of their characteristics should not be confused with the hemolytic types of human origin. Such organisms have been studied by Salter.[1]

In recent years tests have been devised which have helped materially to separate the human and bovine hemolytic streptococci. On blood plates, both produce hemolytic zones about the colonies, but usually the zone of hemolysis is larger about the colonies of the human type. Careful measurement of their hemolytic activity in our laboratories has shown that the human type is approximately 100 times as active as the bovine streptococcus. This extreme difference is easily overlooked on plates, but the difference has been recognized by Brown,[2]

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[1] Am. Jour. Hyg., 1921, 1, p. 154.
[2] Jour. Exper. Med., 1920, 31, p. 35.

who has made use of it in a method of testing for hemolysis in order to separate the two types.

Another test which has served to distinguish between hemolytic streptococci of human and bovine origin is the difference in their final hydrogen-ion concentration. The difference in final P_H among streptococci as pointed out by one of us (Ayers) [3] was applied by Avery and Cullen [4] to differentiate between the hemolytic human and bovine types. The bovine type gives a higher acidity in dextrose broth than the human type and the difference in P_H, while small, is quite definite and characteristic in the proper medium.

There are two tests, therefore, the hemolysis test of Brown and the final hydrogen-ion concentration, which can now be used to distinguish between the human and bovine types of hemolytic streptococcus. These tests are valuable, but of course the difference in hemolytic activity and acid-producing power may be considered only a matter of difference in degree and not a fundamental difference. While we do not believe that such a view should be taken, it seemed desirable to find other differences, if possible.

HYDROLYSIS OF SODIUM HIPPURATE

In another paper [5] we mentioned the fact that certain streptococci gave indication of fermenting organic acids, and further work has given definite proof. While engaged in this study it was found by one of us (Rupp) that one of our cultures of hemolytic streptococci of human origin did not hydrolyze sodium hippurate, while a culture of bovine origin split it into benzoic acid and glycocoll. The significance of this was at once obvious, and more cultures were studied and the same results obtained.

There is nothing new in the hydrolysis of sodium hippurate by bacteria, for it was observed as early as 1864 by Van Tieghem [6] and since that time numerous investigators have observed this action by various bacilli and cocci. After the completion of our work a paper by Stapp [7] was found in which he refers to some work by Crisafulli in 1895 which showed that Streptococcus erysipelatis could split sodium hippurate. The original paper by Crisafulli has not been seen by us.

[3] Jour. Bacteriol., 1916, 1, p. 84; Jour. Infect. Dis., 1918, 23, p. 290.
[4] Jour. Exper. Med., 1919, 29, p. 215.
[5] Jour. Infect. Dis., 1921, 29, p. 235.
[6] Compt. Rend. Acad. Sc., 1864, 58, p. 210.
[7] Centralbl. f. Bakteriol., II, 1920, 51, p. 11.

In order to show the hydrolysis of sodium hippurate two cultures were selected, A-34 of human origin and 90H-1 from the udder of a cow. These cultures were grown in the following medium for 7 days at 37 C. with and without sodium hippurate:

10 gm. peptone (Parke-Davis)
5 gm. pepsin
0.03 gm. calcium chloride
1 drop of 1 per cent. solution of ferric chloride
1000 c c of distilled water
NaOH to give PH 7.1

After incubation the cultures were made acid, then distilled, and the volatile acid titrated. The amino-nitrogen was determined by formol titration.

From table 1 it will be seen that the streptococcus of human origin A-34 gave practically the same results with and without the sodium hippurate. But culture 90H-1 from the udder of a cow showed a large increase in volatile acid and in amino-nitrogen in the medium with sodium hippurate. Tests showed that about 96% of the volatile acidity from this medium was benzoic acid. Since hippuric acid is easily decomposed by chemical agents, by an enzyme of the kidney (histoenzym), and by bacteria, into benzoic acid and glycocoll, an increase in amino-nitrogen should be expected. Such was the case, as may be seen from the table.

TABLE 1

ACTION OF REPRESENTATIVE CULTURES OF HEMOLYTIC STREPTOCOCCI OF HUMAN AND BOVINE ORIGIN ON SODIUM HIPPURATE

Culture of Hemolytic Streptococcus	No Hippurate		1% Hippurate	
	C c N/10 Volatile Acid*	Amino-N Mg. per 100 C c Excess over Control	C c N/10 Volatile Acid*	Amino-N Mg. per 100 C c Excess over Control
A-34 (of human origin).....	7.72	15.12	8.21	15.40
90H-1 (of bovine origin).....	6.03	8.68	53.35	69.73

* Volatile acid in 1,000 c c of distillate from 100 c c of medium.

This hydrolysis may be expressed as follows:

Hippuric Acid. Benzoic Acid. Glycocoll.

$$\left.\begin{matrix} C_6H_5CO. \\ +\ OH \end{matrix}\right| \left.\begin{matrix} NH.CH_2COOH \\ H \end{matrix}\right\} = C_6H_5COOH + NH_2CH_2COOH$$

The amount of sodium hippurate used was 1%, and to show the approximate amount hydrolyzed the following calculation has been made from the results obtained with culture 90H-1: The excess of amino-nitrogen (69.73 mg.) corresponds to 373.9 mg. of glycocoll,

while the excess of volatile acid in 1,500 c c of distillate (50.29 c c $\frac{N}{10}$) corresponds to 613.8 mg. benzoic acid. In order to form hippuric acid 373.9 mg. glycocoll would require 608.1 mg. benzoic acid. This shows that the hydrolyzed products are present in the proportion to form hippuric acid and furthermore that, in this case, the hippuric acid was completely hydrolyzed.

RATE OF HYDROLYSIS OF SODIUM HIPPURATE

The rate of the hydrolysis and its relation to simple and complex mediums was of interest because of the effect on the development of a satisfactory test. It was determined therefore in a simple peptone medium and also in a more complicated broth medium containing dextrose.

Peptone Medium

10.0 gm. peptone (Parke-Davis)
1.5 gm. potassium diabasic phosphate
10.0 gm. sodium hippurate
1000 c c distilled water
PH 7.2

Dextrose Broth Medium

1000 c c infusion broth
10.0 gm. peptone (Parke-Davis)
1.5 gm. potassium diabasic phosphate
10.0 gm. sodium hippurate
2.0 gm. dextrose
PH 7.2

Flasks containing 100c c of medium were prepared and inoculated with culture 90H-1, a bovine udder type of hemolytic streptococcus.

TABLE 2

RATE OF HYDROLYSIS IN SIMPLE AND COMPLEX MEDIUMS

Days	Volatile Acid from Hippurate	
	Peptone and Sodium Hippurate C c	Infusion Dextrose Broth and Sodium Hippurate C c
1	25.72*	41.21
2	42.93	41.29
3	42.74	41.40
4	42.47	42.20
5	42.65	41.43

* C c N/10 volatile acid in 1,000 c c distillate from 100 c c medium.

The volatile acid produced in medium without sodium hippurate was determined and subtracted, so table 2 shows the amount of volatile acid (benzoic acid) from the hippurate during 5 days of incubation at 37° C. It will be observed that the hydrolysis proceeded at a greater rate in the dextrose broth medium during the first 24 hours of incubation, but after that the results were practically identical. This was undoubtedly due to the more rapid growth in the more complex medium during the first 24 hours.

From these results it is evident that the presence of dextrose or beef broth does not interfere with the hydrolysis, and that 48-hours' incubation is sufficient, at least for the culture studied.

INFLUENCE OF HYDROGEN-ION CONCENTRATION ON THE HYDROLYSIS

The well-known influence of hydrogen-ion concentration in accelerating or retarding enzyme action suggested the possibility of similar effects on the splitting of sodium hippurate.

In order to determine the effect of acidity on the hydrolysis two mediums were prepared, one being heavily and the other lightly buffered. The sugar content was also adjusted so that in the lightly buffered medium the acidity would rapidly increase.

The composition of the mediums was as follows:

Medium A	Medium B
1,000 c c infusion broth	1,000 c c infusion broth
10 gm. peptone (Parke-Davis)	10 gm. peptone (Parke-Davis)
10 gm. sodium hippurate	10 gm. sodium hippurate
2 gm. dextrose	5 gm. dextrose
10 gm. potassium diabasic phosphate	P_H 7.2
P_H 7.2	

Flasks containing 100 c c of these mediums were inoculated with culture A-34, a hemolytic streptococcus of human origin, and culture 90H-1 of bovine origin.

TABLE 3

EFFECT OF THE DEVELOPMENT OF ACIDITY IN THE MEDIUM ON THE HYDROLYSIS

Medium	Culture	No Hippurate		Hippurate		Volatile Acid from Hippurate C c*
		N/10 Volatile Acid, C c*	P_H	N/10 Volatile Acid, C c*	P_H	
A	A-34	22.76	6.4	22.60	6.4	0.0
	90H-1	19.44	6.4	64.15	6.4	44.71
B	A-34	12.35	4.9	12.30	5.1	0.0
	90H-1	11.65	4.6	56.53	5.1	44.88

* In 1,000 c c distillate from 100 c c culture medium.

The results given in table 3 again show that A-34 did not split sodium hippurate and that the increase of acidity in the medium had no effect on the hydrolysis by culture 90H-1. In medium A, 44.71 c c $\frac{N}{10}$ volatile acid (benzoic acid) was formed from the hippurate, while the acidity had increased from P_H 7.2 to P_H 6.4. In medium B, the acidity increased from P_H 7.2 to P_H 5.1, and 44.88 c c $\frac{N}{10}$ volatile acid was found.

This experiment, of course, does not show directly the effect of acidity on the hydrolyzing enzyme, because the original P_H was on the alkaline side and the hydrolyzing action may have taken place during the bacterial development and while hydrogen-ion concentration was increasing.

The experiments show, however, that the development of acidity in the medium did not interfere with the hydrolysis of the hippurate and consequently need not be given consideration in connection with tests for this reaction.

The effect of an alkaline reaction was determined in a little different manner in the following medium:

10 gm. peptone (Parke-Davis)
5 gm. pepsin
1 gm. sodium chloride
0.03 gm. calcium chloride
1,000 c c distilled water
NaOH to give the required P_H

Two series of 2 flasks each were prepared, each flask containing 100 c c of this medium, one series without sodium hippurate and the other with 1% of this substance. In each series one of the flasks was adjusted to P_H 8.0 and the other P_H 9.0, and all were inoculated with culture 90H-1. Table 4 shows no growth occurred at P_H 9.0 while the alkaline reaction of P_H 8.0 showed no influence on the hydrolysis. The amount of $\frac{N}{10}$ volatile acid found was 39.0 c c, as compared with the usual amounts which ranged generally from 40 to 44 c c. While the amount in this reaction was slightly lower, it will be seen that only 500 c c of distillate was obtained, against the usual volume of 1,000 c c.

TABLE 4

EFFECT OF INITIAL ALKALINE REACTION ON HYDROLYSIS

Culture	No Hippurate		1% Hippurate	
	P_H 8.0	P_H 9.0	P_H 8.0	P_H 9.0
90H-1	C c* 3.76	C c* No growth	C c* 39.00 P_H after growth 8.0	C c* No growth

* N/10 volatile acid in 500 c c distillate from 100 c c of medium.

These experiments indicate clearly that if a medium is suitable for a good growth of a streptococcus capable of splitting sodium hippurate, its composition or reaction has no effect on the hydrolysis.

APPLICATION OF THE HYDROLYSIS OF SODIUM HIPPURATE TO THE DIFFERENTIATION OF HEMOLYTIC STREPTOCOCCI OF HUMAN AND BOVINE ORIGIN

The value of any tests which will help to differentiate between hemolytic streptococci of human and bovine origin will be readily appreciated by a careful comparison of the cultural characteristics shown in tables 5 and 6. The greatest difference was the more active hemolysis of the human types on blood-agar plates. Both human and bovine cultures showed the beta type of hemolysis. Practically the only other difference was in the fermentation of salicin and mannite. These differences in fermentation were not at all constant and are apparently of no real value.

TABLE 5

CULTURAL CHARACTERISTICS OF HEMOLYTIC STREPTOCOCCI (BETA TYPE) FROM THE UDDER OF COWS

Culture No.	Fermentations							NH_3 from Peptone	CO_2 from Peptone	CO_2 from Dextrose
	Dextrose	Lactose	Saccharose	Salicin	Mannite	Raffinose	Inulin			
8H	+	+	+	+	—	—	—	+	+	—
8H-1	+	+	+	+	—	—	—	+	+	—
8H-2	+	+	+	+	—	—	—	+	+	—
11H	+	+	+	—	—	—	—	+	+	—
16H-2	+	+	+	—	—	—	—	+	+	—
16H-3	+	+	+	—	—	—	—	+	+	—
20H	+	+	+	+	—	—	—	+	+	—
20H-1	+	+	+	+	—	—	—	+	+	—
20H-3	+	+	+	+	—	—	—	+	+	—
25H-1	+	+	+	—	—	—	—	+	+	—
28H-1	+	+	+	+	—	—	—	+	+	—
28H-2	+	+	+	+	—	—	—	+	+	—
28H-3	+	+	+	+	—	—	—	+	+	—
28H-4	+	+	+	+	—	—	—	+	+	—
32H-3	+	+	+	+	—	—	—	+	+	—
32H-4	+	+	+	+	—	—	—	+	+	—
32H-x	+	+	+	+	—	—	—	+	+	—
37H-4	+	+	+	+	—	—	—	+	+	—
37H-S	+	+	+	+	—	—	—	+	+	—
38H	+	+	+	—	—	—	—	+	+	—
39H-3	+	+	+	—	—	—	—	+	+	—
41H-1	+	+	+	+	—	—	—	+	+	—
41H-2	+	+	+	+	—	—	—	+	+	—
41H-3	+	+	+	+	—	—	—	+	+	—
41H-x	+	+	+	+	—	—	—	+	+	—
42H-3	+	+	+	—	—	—	—	+	+	—
54H	+	+	+	—	—	—	—	+	+	—
54H-1	+	+	+	—	—	—	—	+	+	—
59H	+	+	+	—	—	—	—	+	+	—
59H-1	+	+	+	—	—	—	—	+	+	—
67H-1	+	+	+	+	—	—	—	+	+	—
80H	+	+	+	—	—	—	—	+	+	—
80H-2	+	+	+	—	—	—	—	+	+	—
83H	+	+	+	—	—	—	—	+	+	—
90H	+	+	+	+	—	—	—	+	+	—
90H-1	+	+	+	+	—	—	—	+	+	—
96H	+	+	+	—	—	—	—	+	+	—
97H	+	+	+	—	—	—	—	+	+	—
103H-2	+	+	+	+	—	—	—	+	+	—
106H-4	+	+	+	—	—	—	—	+	+	—
108H-2	+	+	+	—	—	—	—	+	+	—
113H-1	+	+	+	—	—	—	—	+	+	—
115H-2	+	+	+	—	—	—	—	+	+	—
126H-1	+	+	+	—	—	—	—	+	+	—

TABLE 6

CULTURAL CHARACTERISTIC OF HEMOLYTIC STREPTOCOCCI (BETA TYPE) OF HUMAN ORIGIN

Culture No.	Source	Fermentations							NH_3 from Peptone	CO_2 from Peptone	CO_2 from Dextrose
		Dextrose	Lactose	Saccharose	Salicin	Mannite	Raffinose	Inulin			
A 32	Sputum, postinfluenzal pneumonia	+	+	+	+	+	−	−	+	+	−
A 34	Abscess in myocardium	+	+	+	+	−	−	−	+	+	−
A 38	Throat, tonsillitis	+	+	+	+	+	−	−	+	+	−
A 42	Throat, measles	+	+	+	+	+	−	−	+	+	−
A 43	Throat, measles	+	+	+	+	−	−	−	+	+	−
A 45	Throat, measles	+	+	+	+	+	−	−	+	+	−
A 46	Throat, measles	+	+	+	+	+	−	−	+	+	−
A 47	Throat, measles	+	+	+	+	+	−	−	+	+	−
A 49	Throat, measles	+	+	+	+	+	−	−	+	+	−
A 51	Throat, measles	+	+	+	+	+	−	−	+	+	−
A 52	Throat, measles	+	+	+	+	−	−	−	+	+	−
A 56	Normal throat	+	−	+	+	−	−	−	+	+	−
A 59	Normal throat	+	−	+	+	−	−	−	+	+	−
A 60	Normal throat	+	+	+	+	−	−	−	+	+	−
A 63	Normal throat	+	+	+	+	−	−	−	+	+	−
A 64	Normal throat	+	+	+	+	−	−	−	+	+	−
A 65	Throat, pharyngitis	+	+	+	+	−	−	−	+	+	−
A 66	Normal throat	+	+	+	+	−	−	−	+	+	−
A 67	Throat, tonsillitis	+	+	+	+	−	−	−	+	+	−
A 69	Throat, bronchitis	+	+	+	+	−	−	−	+	+	−
A 73	Throat, tonsillitis	+	+	+	+	−	−	−	+	+	−
A 74	Throat, normal	+	+	+	+	−	−	−	+	+	−
A 78	Throat, normal	+	+	+	+	−	−	−	+	+	−
A 79	Throat, normal	+	+	+	+	−	−	−	+	+	−
A 80	Throat, normal	+	+	+	+	−	−	−	+	+	−
A 81	Throat, normal	+	+	+	+	−	−	−	+	+	−
A 82	Throat, normal	+	+	+	+	−	−	−	+	+	−
A 94	Throat, normal	+	+	+	+	−	+	−	+	+	−
A 95	Hemolytic streptococcus, army strain	+	+	+	+	−	−	−	+	+	−
R 2	Lung necropsy	+	+	+	+	−	−	−	+	+	−
R 24	Bronchopneumonia	+	+	+	+	−	−	−	+	+	−
R 66	Throat, measles	+	+	+	+	+	−	−	+	+	−
R 271	Blood, septicemia	+	+	+	+	−	−	−	+	+	−

The ability of these hemolytic cultures from human and bovine sources to hydrolyze sodium hippurate was determined in the most simple medium which would support growth, in order to minimize the formation of volatile acid from anything but the hippurate. For this purpose the following peptone medium was quite satisfactory:

10 gm. peptone (Parke-Davis)
10 gm. sodium hippurate
1.5 gm. potassium diabasic phosphate
1,000 c c distilled water
Reaction adjusted to PH 7.2

The medium was put up in 100 c c amounts in flasks, and after inoculation was incubated 7 days at 37 C. Growth was slow in starting with some cultures and moderate in amount in all cases. Therefore it was considered advisable to allow 7 days for incubation. After incubation the cultures were made acid, and the volatile acidity was determined in 500 c c of distillate from 100 c c of culture.

All our 33 hemolytic cultures of human origin and 44 hemolytic cultures of the beta type of bovine origin were examined for their ability to split sodium hippurate. From the results in table 7 it is

evident that the hydrolysis of the hippurate by the hemolytic streptococci of bovine origin separated them perfectly from those of human origin. All of the bovine types split the hippurate, while none of the

TABLE 7

DIFFERENTIATION OF HEMOLYTIC STREPTOCOCCI OF HUMAN AND BOVINE ORIGIN BY MEANS OF THE HYDROLYSIS OF SODIUM HIPPURATE SHOWING HOW A POSITIVE REACTION CORRELATES WITH A LOW PH

Hemolytic Streptococci, Udders of Cows				Hemolytic Streptococci, Human Sources			
Culture No.	C c N/10 Volatile Acid	Hydrolysis	PH Dextrose Yeast Broth	Culture No.	C c N/10 Volatile Acid	Hydrolysis	PH Dextrose Yeast Broth
8H	40.52*	+	4.5	A 32	3.65*	—	5.5
8H-1	41.51	+	4.5	A 34	3.37	—	5.4
8H-2	44.73	+	4.5	A 38	3.50	—	5.3
11H	41.17	+	4.5	A 42	3.31	—	5.4
16H-2	38.00	+	4.5	A 43	3.64	—	5.4
16H-3	39.08	+	4.5	A 45	3.19	—	5.5
20H	39.51	+	4.5	A 46	3.50	—	5.5
20H-1	42.36	+	4.6	A 47	3.52	—	5.5
20H-3	40.71	+	4.5	A 49	3.31	—	5.5
25H-1	41.42	+	4.5	A 51	3.49	—	5.5
28H-1	40.14	+	4.5	A 52	2.84	—	5.5
28H-2	44.14	+	4.6	A 56	3.53	—	5.3
28H-3	39.61	+	4.5	A 59	3.22	—	5.4
28H-4	39.96	+	4.5	A 60	2.90	—	5.5
32H-3	43.89	+	4.5	A 63	3.24	—	5.5
32H-4	35.64	+	4.5	A 64	3.11	—	5.6
32H-x	42.27	+	4.6	A 65	3.40	—	5.6
37H-4	42.12	+	4.5	A 66	2.47	—	5.6
37H-5	36.22	+	4.5	A 67	3.05	—	5.5
38H	41.01	+	4.6	A 69	3.10	—	5.6
39H-3	39.45	+	4.5	A 73	3.13	—	5.5
41H-1	42.52	+	4.5	A 74	3.37	—	5.5
41H-2	44.27	+	4.5	A 78	3.08	—	5.4
41H-3	39.73	+	4.5	A 79	3.31	—	5.3
41H-x	41.83	+	4.5	A 80	3.27	—	5.6
42H-3	40.24	+	4.6	A 81	3.54	—	5.6
54H	42.72	+	4.5	A 82	3.56	—	5.6
54H-1	43.10	+	4.5	A 94	1.44	—	4.6
59H	40.32	+	4.5	A 95	2.60	—	5.5
59H-1	41.23	+	4.5	R 2	3.39	—	5.4
67H-1	38.36	+	4.5	R 24	1.36	—	5.5
80H	41.02	+	4.5	R 66	3.46	—	5.6
80H-2	41.79	+	4.5	R 271	2.32	—	5.4
83H	39.03	+	4.5				
90H	43.07	+	4.5	A 34	No hippurate 4.86		
90H-1	36.69	+	4.5				
96H	44.70	+	4.5				
97H	38.63	+	4.5				
103H-2	40.08	+	4.6				
106H-4	39.07	+	4.5				
108H-2	40.07	+	4.5				
113H-1	20.07	+	4.5				
115H-2	37.28	+	4.5				
126H-1	41.04	+	4.6				
90H-1	No hippurate 3.28						

* C c N/10 volatile acid in 500 c c distillate from 100 c c of medium.

human type possessed this ability. The hemolytic streptococci from both human and bovine sources produced from 3 to 4 c c $\frac{N}{10}$ volatile acid in the medium without sodium hippurate. When there was a hydrolysis the volatile acidity was increased to about 40 c c; both qualitative and quantitative tests showed the excess to be benzoic acid.

A further study of the results shows that all of the bovine types reached a final hydrogen-ion concentration is dextrose yeast broth of P_H 4.5-4.6, while all the human types reached only P_H 5.3-5.6, with one exception. This exception is interesting, since by P_H it might be considered a bovine type, or by the lack of power to hydrolyze hippurate it could be considered a human type. This culture (A-94) was isolated from a normal throat and varied from the other human types in its ability to ferment raffinose. It does not appear to be of the ordinary human type either in its fermentations or in final P_H, yet it is clearly not a bovine udder organism because of its inability to hydrolyze sodium hippurate.

The use of sodium hippurate should materially assist in the proper placing of cultures of this type which are found in normal throats.

Our results indicate that the hemolytic B types of streptococci of human origin can be separated from similar types of bovine origin by the inability of the human type to hydrolyze sodium hippurate. We realize, however, that a larger number of cultures must be examined before definite statements regarding this can be made.

TESTS FOR THE HYDROLYSIS OF SODIUM HIPPURATE

In our work we used a simple peptone medium and distilled each culture for volatile acidity in order to determine the hydrolysis. This process has the advantage of giving clear-cut results, but it is too long for routine work.

Several rapid tests have therefore been worked out and are suggested for the routine examination of cultures. It is important that a simple medium be used, which will show good growth, and for this purpose the following medium is recommended.

Peptone Pepsin Medium

10 gm. peptone (Parke-Davis)	1 drop of 1 per cent. ferric chloride solution
5 gm. pepsin	1,000 c c distilled water
.03 gm. calcium chloride	NaOH to give P_H 7.1
10 gm. sodium hippurate	

If cultures are found which will not grow in this medium, the usual beef-infusion peptone broth with or without dextrose may be used, but the tests are not quite so satisfactory with this complex medium.

The presence of benzoic acid in the medium can be demonstrated by the addition of either a ferric-chloride solution or an inorganic acid, the ferric-chloride test being the more delicate of the two. The protein, the hippurate, and the benzoate are precipitated by ferric chloride, the difference between them being that the protein and the hippurate pre-

cipitates are more readily soluble in an excess of ferric chloride than the benzoate. If, therefore, we add a definite amount of ferric chloride solution to a fixed quantity of the medium, the reaction can be so balanced that the protein and hippurate precipitate redissolve in the excess of the reagent while the benzoate remains as an insoluble precipitate.

Ferric-Chloride Test.—The amount of ferric chloride required depends on the medium used. In the case of the peptone pepsin medium, 0.5 c c of a 7% solution of ferric chloride is added to 2 c c of the medium. When it is thoroughly shaken, an insoluble precipitate remains in the mixture if the hippurate has been split into benzoate and glycocoll, whereas the mixture becomes clear on standing 5 or 10 minutes if the hippurate has not been hydrolyzed. The precipitate of ferric benzoate is well marked when one-fifth or more of the hippurate has been split, while only a turbidity is produced when less has been hydrolyzed.

Udder hemolytic streptococci hydrolyze the hippurate completely or nearly so, and the test is sharp and distinct. A slight opalescence is generally present in the medium containing only hippurate which is not acted on by the hemolytic streptococci of human origin, and this becomes somewhat more marked when some of the growth is transferred to the test tube, but neither turbidity nor precipitate is produced.

If the medium contains phosphates, it is necessary to add a small amount of hydrochloric acid to the ferric-chlorid solution, so as to redissolve the phosphate of iron formed.

In the case of a beef-infusion peptone medium, it is necessary to use a 12% ferric chloride solution containing from 2.0 to 2.5 c c concentrated hydrochloric acid per liter, and it requires a longer time for the medium to become clear when the hippurate has not been hydrolyzed.

ACID TEST

On adding an excess of an inorganic acid to the medium, the benzoic acid separates as a white crystalline precipitate. About one-half of the hippurate must be split in order to give a well-marked precipitate, the protein in the solution preventing the separation of the acid if present in only small amounts.

Test: Five-tenths c c of 50% sulphuric acid is added to 2 c c of the medium; the mixture is well shaken and allowed to stand. In cultures of hemolytic streptococci of bovine origin a large amount of benzoic acid is always present.

Hydrolysis Indicated by Increase in Amino-Nitrogen.—As hippuric acid is split into benzoic acid and glycocoll, there should be an increase in the amino-nitrogen content of the medium unless the glycocoll is utilized by the bacteria.

Our results idicate that there is such an increase with the streptococci during hydrolysis. A formol titration therefore shows a considerable increase in amino-nitrogen in cultures which hydrolyze hippuric acid, and this increase may be as high as eight times the amount found in control cultures without the hippurate, as is shown in table 1.

This large increase in amino-nitrogen therefore constitutes another test for the hydrolysis.

SUMMARY

It has been shown that hippuric acid is hydrolyzed by the 44 hemolytic streptococci from the udders of cows, but not by the 33 hemolytic streptococci of human origin which are in our collection of cultures.

As much as 1% of hippurate may be split into benzoic acid and glycocoll.

The hydrolysis is not affected by the hydrogen-ion concentration of the medium, at least under the experimental conditions of our work.

The composition of the medium does not appear to affect the hydrolysis, provided it is suitable for the growth of the streptococci.

Simple tests have been devised for the detection of the hydrolysis routine work.

The hydrolysis of sodium hippurate seems to separate the hemolytic beta streptococci of the bovine udder from those of human origin, but should be used at present only with beta hemolytic types. It is hoped this reaction will be equally valuable after large numbers of cultures have been examined.

Particular attention is called to the fact that the usefulness of the hydrolysis of sodium hippurate is discussed only in its relation to the beta hemolytic streptococci of human and bovine origin. Our studies have shown that the ability of streptococci to split sodium hippurate is not limited to the hemolytic types. Some of the alpha types from the udder of the cow do not produce the hydrolysis, while, on the other hand, the hydrolyzing property is common among the lactic type of streptococci. The test must not be applied indiscriminately, therefore, to all groups of streptococci.